CANADIAN JOURNAL OF RESEARCH

VOLUME 26 JUNE, 1948 NUMBER 3

— SECTION E —

MEDICAL SCIENCES

Contents

Page

A Method of Typing *Haemophilus influenzae* by the Precipitin Reaction—*Catherine F. C. MacPherson* - - - - - - - 197

The Inhibition of Hyaluronidase by Sodium Salicylate and its Possible Metabolites—*Julius Lowenthal and Arthur Gagnon* - 200

Glycosuria in Phlorizinized Rats Depleted of Pyridoxine—*Edouard Pagé and R. Gingras* - - - - - - - - - - 206

The Diet and Hormonally Induced Nephrosclerosis—*E. C. Hay, J. L. Prado, and H. Selye* - - - - - - - - - - 212

NATIONAL RESEARCH COUNCIL
OTTAWA, CANADA

CANADIAN JOURNAL OF RESEARCH

The *Canadian Journal of Research* is issued in six sections, as follows:

A. Physical Sciences	D. Zoological Sciences
B. Chemical Sciences	E. Medical Sciences
C. Botanical Sciences	F. Technology

For the present, Sections A, C, D, and E are to be issued six times annually, and Sections B and F, twelve times annually, each under separate cover, with separate pagination.

The *Canadian Journal of Research* is published by the National Research Council of Canada under authority of the Chairman of the Committee of the Privy Council on Scientific and Industrial Research. The *Canadian Journal of Research* is edited by a joint Editorial Board consisting of members of the National Research Council of Canada, the Royal Society of Canada, and the Chemical Institute of Canada.

Sections B and F of the *Canadian Journal of Research* have been chosen by the Chemical Institute of Canada as its medium of publication for scientific papers.

EDITORIAL BOARD

Representing
NATIONAL RESEARCH COUNCIL

DR. A. R. GORDON, (*Chairman*),
Head, Department of Chemistry,
University of Toronto, Toronto.

DR. C. H. BEST,
The Banting and Best Department
of Medical Research,
University of Toronto, Toronto.

DR. ROBERT NEWTON,
President, University of Alberta,
Edmonton, Alta.

DR. G. H. HENDERSON,
Professor of Mathematical
Physics,
Dalhousie University, Halifax.

Representing
ROYAL SOCIETY OF CANADA

DR. A. NORMAN SHAW,
Chairman, Department of Physics
McGill University, Montreal.

DR. J. W. T. SPINKS,
Department of Chemistry,
University of Saskatchewan,
Saskatoon.

Section III

DR. E. HORNE CRAIGIE,
Department of Zoology,
University of Toronto,
Toronto.

DR. H. S. JACKSON,
Head, Department of Botany,
University of Toronto, Toronto.

Section V

Ex officio

DR. LÉO MARION, Editor-in-Chief,
Division of Chemistry,
National Research Laboratories,
Ottawa.

DR. H. H. SAUNDERSON,
Director, Division of
Information Services,
National Research Laboratories,
Ottawa.

Representing
THE CHEMICAL INSTITUTE OF CANADA

DR. R. V. V. NICHOLLS,
Associate Professor of Chemistry,
McGill University,
Montreal.

EDITORIAL COMMITTEE

Editor-in-Chief, DR. LÉO MARION
Editor, Section A, DR. A. NORMAN SHAW
Editor, Section B, DR. J. W. T. SPINKS, DR. R. V. V. NICHOLLS
Editor, Section C, DR. H. S. JACKSON
Editor, Section D, DR. E. HORNE CRAIGIE
Editor, Section E, DR. J. B. COLLIP
Editor, Section F, DR. J. A. ANDERSON, DR. R. V. V. NICHOLLS, DR. A. NORMAN SHAW

Manuscripts should be addressed:

Editor-in-Chief,
Canadian Journal of Research,
National Research Council, Ottawa, Canada.

Canadian Journal of Research

Issued by THE NATIONAL RESEARCH COUNCIL OF CANADA

VOL. 26, SEC. E. JUNE, 1948 NUMBER 3

A METHOD OF TYPING *HAEMOPHILUS INFLUENZAE* BY THE PRECIPITIN REACTION[1]

BY CATHERINE F. C. MACPHERSON[2]

Abstract

A method of typing *H. influenzae* by the precipitin reaction is described. The procedure consists of dissolving the bacterial culture in 90% phenol to destroy the specificity of the somatic proteins followed by alcoholic precipitation of the denatured proteins and type specific polysaccharide. The carbohydrate is extracted from the precipitate with saline and portions of the saline extract added to samples of the six type specific antisera. A positive test is indicated only by a marked turbidity, which develops within five minutes after mixing and denotes the extraction of at least 0.01 mgm. of polysaccharide. A strong positive test is, of course, regularly obtained in the case of cultures shown to be encapsulated by the usual capsular swelling technique. However, the method was devised as an attempt to ascertain whether non-typable (by the capsular swelling technique) derivatives of strains of known type or non-typable, but suspected respiratory pathogens, isolated from clinical material, contained detectable quantities of any of the known type specific polysaccharides.

Introduction

There are now six recognized types of pathogenic *Haemophilus influenzae* (5). These pathogenic strains, in contrast to the nonpathogenic forms, produce an iridescent growth on Levinthal or Fildes agar and have a well defined capsule. The specific capsular substances of Types *a*, *b*, *c*, *d*, and *f* have been shown to be polysaccharides. Some of the chemical, physical, and immunochemical properties of these carbohydrates have been recorded (4).

In the course of immunochemical studies on the somatic proteins of *H. influenzae*, it was noted that extracts of non-encapsulated derivatives of Type *b* meningeal strains contained appreciable quantities of Type *b* polysaccharide. One derivative strain that was not iridescent and not encapsulated has been subcultured repeatedly on Fildes agar over a period of three years and continued regularly to produce the capsular substance.

The frequency with which one encounters colonies of non-encapsulated organisms in cultures of pathogenic *H. influenzae* under laboratory conditions subsequent to isolation from clinical material is well known. Thus it was considered that a method by which minute amounts of capsular substance could be isolated and identified by the precipitin reaction would be useful as

[1] *Manuscript received February 6, 1948.*

Contribution from the Department of Bacteriology and Immunology, McGill University, Montreal, Que. The work reported in this communication was supported by grants from the Commonwealth Fund.

[2] *Research Fellow, Department of Bacteriology and Immunology, McGill University, Montreal, Que.*

an adjunct to the capsular swelling technique in identifying a poorly encapsulated strain as well as in ascertaining the origin of non-encapsulated derivative strains in which the transition from S⟶R was not complete.

Method

The method finally devised is as follows. To the yield of heavy, confluent 18 hr. growth from one Fildes or Levinthal agar plate suspended in 1.5 ml. of 1 *M* sodium acetate are added five volumes of 95% ethyl alcohol. After centrifugation, the supernatant is discarded, the precipitate (measuring about 0.15 ml. in volume) smoothed out and next dissolved by the addition of 1.0 ml. of 90% phenol for 15 min. Phenol denatures the bacterial proteins and renders them insoluble in saline but does not injure the specific polysaccharides. Then 0.1 ml. of 20% sodium acetate and 5 ml. of chilled ethyl alcohol are added with thorough mixing and the tubes left at 0° C. for at least one hour. The polysaccharides are less soluble in alcohol at 0° C. than at room temperature. Thus for good recovery of polysaccharide it is advisable to maintain all alcoholic mixtures at this lower temperature. The bulky precipitate of denatured protein and polysaccharide is centrifuged down, washed twice with 2 ml. portions of chilled alcohol to remove all but traces of phenol, and finally extracted with 1.0 ml. of saline. To perform the test, 0.1 ml. of clear extract is added to 0.1 ml. portions of each of the six type specific antisera in 50 × 6 mm. tubes and the tubes are read within five minutes after mixing. The antisera should contain at least 0.3 mgm. of precipitin nitrogen per ml. (1, 2, 3, 4). Only immediate marked turbidity is considered as a positive test and this depends upon the extraction of about 0.01 mgm. of polysaccharide.

After 15 to 30 min. a slight cloudiness develops in those tubes that contain anti *c*, *d*, *e*, or *f* antisera. This precipitation is due to a reaction between a species specific protein, which escapes denaturation by phenol and the species specific antibody that comprises a relatively large proportion of the total antibody in Types *c*, *d*, *e*, and *f* antisera.

Results

At least one non-typable variant of each of the six kinds of encapsulated influenza strains has been examined by this method. The Type *b* variants regularly gave a positive test for type specific carbohydrate whereas Type *a*, *c*, *d*, *e*, and *f* variants usually yielded negative or weakly positive results. Studies are in progress on additional derivatives of encapsulated strains.

The knowledge that some non-encapsulated strains that were variants of known encapsulated strains contained detectable amounts of type specific polysaccharide suggested that the method described above might be used to throw some light on the question as to whether or not the large numbers of non-encapsulated, non-iridescent strains of *H. influenzae* that are often isolated from clinical material are derived from encapsulated strains.

To date the method has been applied to a group of 20 such strains. Ten of these were isolated from normal throats and 10 from cases of respiratory infections. In all, the results were negative.

Acknowledgments

The author is indebted to Dr. Hattie Alexander, College of Physicians and Surgeons, Columbia University, for the cultures of *H. influenzae* used in this study, and to Dr. E. G. D. Murray, McGill University, for helpful suggestions and criticism.

References

1. HEIDELBERGER, M. and KENDALL, F. E. J. Exptl. Med. 50 : 809. 1929.
2. HEIDELBERGER, M. and KENDALL, F. E. J. Exptl. Med. 61 : 559. 1935.
3. HEIDELBERGER, M., KENDALL, F. E., and SOO HOO, C. M. J. Exptl. Med. 58 : 137. 1933.
4. MACPHERSON, C. F. C., HEIDELBERGER, M., ALEXANDER, H. E., and LEIDY, G. J. Immunol. 52 : 207. 1946.
5. PITTMAN, M. J. Exptl. Med. 53 : 471. 1931.

THE INHIBITION OF HYALURONIDASE BY SODIUM SALICYLATE AND ITS POSSIBLE METABOLITES[1]

By Julius Lowenthal[2] and Arthur Gagnon[3]

Abstract

It has been shown that salicylic acid and gentisic acid, a substance formed in the organism from salicylic acid, have no influence on the activity of hyaluronidase *in vitro*. However carboxy-*p*-benzoquinone, the quinone corresponding to gentisic acid, was found to inhibit the enzyme *in vitro*.

Salicylic acid and its derivatives have been used for more than 50 years in the treatment of rheumatic fever. Despite the fact that during this period salicylates have been one of the most frequently investigated drugs, the mechanism underlying their therapeutic effects has remained obscure. This state of affairs has been due, to a large extent, to our ignorance of the etiology of rheumatic fever, and this naturally has had a bearing on all these investigations.

Recently a number of facts have become known that seem to give some indications as to the nature of rheumatic fever, as well as the mode of action of salicylates in the treatment of this disease.

It has been known for some time that extracts from such sources as invasive bacteria (like hemolytic streptococci Group A (5, 16)), testes (12), poisonous insects, and snake venoms (7) increase the permeability of the skin. This can be demonstrated by injecting these extracts (spreading factors) together with suitable indicators (India ink) intradermally in experimental animals and comparing the area of diffusion with that of the simultaneously injected indicator minus the spreading factor. Working along chemical lines, a number of investigators have been able to isolate from these spreading factors an enzyme, hyaluronidase, which acts specifically on a mucopolysaccharide, hyaluronic acid (20, 3). This polysaccharide is found in the skin (18), umbilical cord, synovial fluid (19), and other sources, and more generally seems to form an essential constituent of the connective tissue. When the action of the enzyme on its substrate is followed *in vitro*, there is first a depolymerization, as can be seen by a decrease in the viscosity and by the loss of the protein precipitating power of hyaluronic acid. This is followed later by complete hydrolysis, as evidenced by increased reducing power. It is precisely in those regions rich in hyaluronic acid that the swelling and lesions, so characteristic of rheumatic fever, are known to occur. Furthermore, it has been

[1] *Manuscript received in original form December 17, 1947, and as revised, March 9, 1948. Contribution from the Department of Physiological Chemistry, University of Montreal, Montreal, Que., with financial assistance from the National Research Council of Canada. Paper based on a thesis submitted to the Faculty of Science in partial fulfilment of the requirements for the degree of Master of Science.*

[2] *Graduate Student.*

[3] *Assistant Professor of Physiological Chemistry.*

observed for some time that rheumatic fever is frequently preceded by streptococcal infections of the throat, from which organism hyaluronidase has been extracted.

Since it is generally held to-day that no causative bacterial agent has ever been isolated from the affected area in rheumatic fever, a possible causal relationship between hyaluronidase and rheumatic fever may tentatively be inferred.

A recent experiment by Guerra (9) has been the starting point for the present work, and seems to be of great significance when viewed in relation to the above-mentioned facts. When hyaluronidase together with India ink was injected into the skin of albino rabbits or human subjects after the intravenous injection of sodium salicylate, there was an inhibition of the spreading. It would appear that this action is specific for salicylates, for when sulphadiazine was tested, no such inhibition was observed. On the basis of Guerra's work, we concluded that if this decrease in diffusion was due to an inhibition of the enzyme hyaluronidase by the salicylate or a product formed from it in the organism, it would perhaps be possible to reproduce this inhibition *in vitro* and in this way obtain more definite information on the mode of action of salicylates in rheumatic fever.

Experimental

Preparation of Test Substances

Extraction of Hyaluronidase from Bovine Testes

The method followed for the extraction of hyaluronidase was essentially that of Hahn (11). The precipitate obtained at 70% ammonium sulphate saturation was suspended in a small amount of water and dialyzed against running water until salt free; after reducing the volume by distillation under reduced pressure at a maximum temperature of 30°, the solution was evaporated to dryness while freezing.

Hyaluronic acid was obtained through the courtesy of Dr. E. Schwenk, Schering Corporation.

The viscosimetric method of following the enzymatic depolymerization of hyaluronic acid by the decrease of the viscosity was used. The experimental arrangement is similar to that of Haas (10) with slight changes in the concentration of enzyme and polysaccharide and in buffer solutions. It is shown in Table I.

Method of calculating the specific viscosity and inhibition

t = time after the addition of hyaluronidase

t_1 = flow time of solution containing buffer enzyme and polysaccharide

t_2 = flow time of solution containing solvents

t_3 = reaction time $= t + 0.5\, t_1$

η = specific viscosity $= \frac{t_1}{t_2} - 1$

R_0 = time to reach half viscosity without inhibitor
R = time to reach half viscosity with inhibitor
I = % of inhibition = $\left(1 - \frac{R_0}{R}\right)$

TABLE I

	Expt. I	Expt. II	Expt. III	Expt. IV	Expt. V	Expt. VI
0.9% NaCl, ml.	1.0	1.0	1.0	1.0	1.0	1.0
Hyaluronidase, mgm. (dissolved in 1 ml. 0.9% NaCl)	—	—	.125	.125	.125	.125
0.5*M* phosphate buffer, pH 7, ml.	1.0	1.0	1.0	—	—	—
1 ml. carboxy-*p*-benzoquinone (dissolved in 0.5*M* phosphate buffer, pH 7), concentration, moles/liter	—	—	—	1×10^{-3}	2.5×10^{-3}	5×10^{-3}
Incubated separately for 10 min. at 26° C.						
0.02 *M* acetate buffer, pH 4.7, ml.	2.0	—	—	—	—	—
Polysaccharide (dissolved in 0.02*M* acetate buffer, pH 4.7) concentration, 2 mgm./ml. ; ml.	—	2.0	2.0	2.0	2.0	2.0
Flow time, sec.	54.3	127.7				
% Inhibition = $100\left(1 - \frac{R_0}{R}\right)$				40.2	60.4	68.2

When sodium salicylate was added to the system in concentration of $10^{-2}M$ and in even higher concentrations, no inhibition was observed. Since this research was started, a number of publications have come to our attention where the same problem was investigated. Pike (22) and Meyer (17) have stated that they failed to observe any inhibition *in vitro* of hyaluronidase by sodium salicylate, while Dorfmann, Reimers, and Ott (6) and Calesnick and Beutner (2) have reported that they were able to produce inhibition by sodium salicylate. Since salicylic acid in higher concentrations is known to be a flocculating and precipitating agent of proteins, it may be that the inactivation observed might have been due to this effect, rather than to a specific inhibition (13).

We concluded, after the negative result with salicylate, that the effect observed by Guerra might have been due to a compound formed from salicylic acid in the organism. One of the outstanding facts in the salicylate therapy of rheumatic fever are the large doses used. Concentrations of 35 to 50 mgm. per ml. of blood has been advocated (4). Studies on the fate

of the compound in the organism show that about 80% is excreted in forms containing intact salicyl groups, while 4 to 8% is converted to gentisic (2,5-dihydroxybenzoic acid) and related compounds (14). The latter seems to be the only new compound formed, since the formation of conjugated product may be considered a general mode of detoxification.

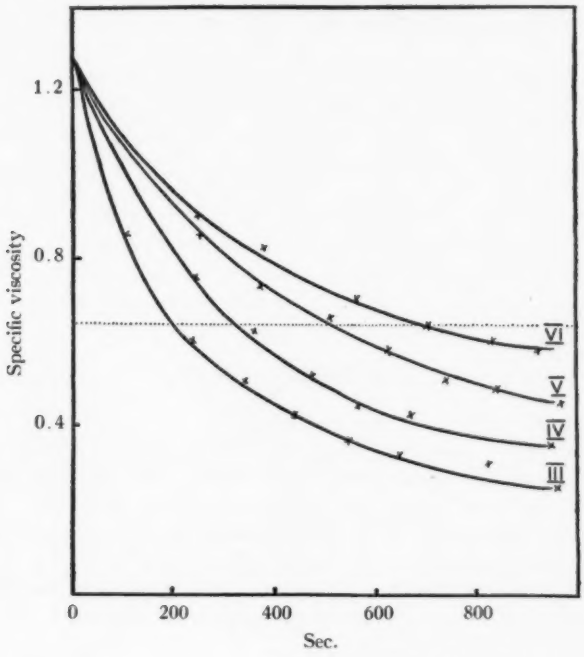

FIG. 1.

We therefore tested the activity of gentisic acid in the same way as that of its precursor, salicylic acid, and this compound was also found to be inactive. Since gentisic acid is a *p*-diphenol, it may be susceptible to further oxidation, the next product of oxidation being the corresponding quinone. In a model experiment with *p*-benzoquinone, a significant inhibition was obtained.

A survey of the literature (21, 23, 2) indicates that several attempts to prepare the quinone corresponding to gentisic acid were unsuccessful. We first tried to obtain this compound by the enzymatic oxidation of gentisic acid. Incubation with peroxidase and polyphenol oxidase showed that gentisic acid was acted upon by these enzymes, but no active substance could be extracted from these mixtures. We finally succeeded in finding a method for the chemical oxidation of gentisic acid to its quinone and this product proved effective as an inhibiting agent, at a concentration of $10^{-3}M$ and higher.

Syntheses

Gentisic acid was prepared after the method of Mauthner (15). It was purified by precipitation from ether with petrol ether (fraction 65° to 95° C.). It gave a fusion point of 204° C. and with ferric chloride a characteristic corn-blue coloration.

Carboxy-*p*-benzoquinone. A method described by Thiele (24) for the preparation of similar unstable quinones by dry oxidation with nitric oxides was used in the preparation of this compound, after attempts to prepare it by other methods failed. Finely powdered gentisic acid is placed on a watch glass in a desiccator over fuming nitric acid. Partial evacuation aids in the evolution of nitric oxides. When the reaction is finished the crystals are placed over soda lime in a vacuum to take off any nitric oxides. The crystalline mass was dissolved in ether, and after filtration and evaporation of the ether, a reddish brown granular mass was obtained. Attempts to find more suitable solvents for obtaining the quinone in a crystalline form were unsuccessful. The compound decomposed without a sharp melting point, and with 2,4-dinitrophenylhydrazine, it yielded a compound that melted at 182° C. It is probable that the quinone was not pure, but still contained unreacted gentisic acid.

General Observations

In discussing the possible significance of the inhibition of hyaluronidase *in vitro* by carboxy-*p*-benzoquinone, it must be borne in mind that the spreading reaction itself is a complex phenomenon (8), depending on many factors, such as age, sex hormones, etc. When the enzymatic activity is followed *in vitro*, conditions are so different that it is hardly warranted to make any definite statement as to the possibility of corresponding mechanism *in vivo*, and carboxy-*p*-benzoquinone has not yet been shown to be formed in the organism after the administration of salicylic acid, although its formation may seem possible.

Moreover, the experimental arrangement in the viscosimetric measurement is such that it is very difficult to draw any definite conclusions as to whether the observed effect is due to a specific inhibition, or to an unspecific denaturation of the enzyme. However, the low concentration of the quinone makes this point unlikely.

We therefore think that it would need further experimental evidence, to correlate our findings with the actual mode of action of salicylates in rheumatic fever. Investigations planned to this end are in progress in our laboratory.

References

1. Brunner, C. Monatsh. 2 : 458. 1881.
2. Calesnick, B. and Beutner, R. Federation Proc. 6 : 314. 1947.
3. Chain, E. and Duthie, E. S. Brit. J. Exptl. Path. 21 : 324. 1940.
4. Coburn, A. F. Bull. Johns Hopkins Hosp. 73 : 435. 1943.
5. Crowley, N. J. Path. Bact. 56 : 27. 1944.
6. Dorfmann, A., Reimers, E. J., and Ott, M. L. Proc. Soc. Exptl. Biol. Med. 64 : 357. 1947.

7. DURAN-REYNALS, F. J. Exptl. Med. 69 : 69. 1939.
8. DURAN-REYNALS, F. Bact. Revs. 6 : 197. 1942.
9. GUERRA, F. J. Pharmacol. 87 : 193. 1946.
10. HAAS, E. J. Biol. Chem. 163 : 63. 1946.
11. HAHN, L. Biochem. Z. 315 : 83. 1943.
12. HOFFMANN, D. C. and DURAN-REYNALS, F. J. Exptl. Med. 53 : 387. 1931.
13. IVANOVICS, G. Z. physiol. Chem. 276 : 33. 1942.
14. KAPP, E. M. and COBURN, A. F. J. Biol. Chem. 145 : 549. 1942.
15. MAUTHNER, N. J. prakt. Chem. 156 : 150. 1940.
16. MCCLEAN, D. J. Path. Bact. 53 : 13. 1941.
17. MEYER, K. Physiol. Revs. 27 : 335. 1947.
18. MEYER, K. and CHAFFEE, E. J. Biol. Chem. 138 : 491. 1941.
19. MEYER, K. and PALMER, J. W. J. Biol. Chem. 107 : 629. 1934.
20. MEYER, K., DUBOS, R., and SMYTH, E. M. J. Biol. Chem. 118 : 71. 1937.
21. NEF, J. U. Ber. 18 : 3496. 1885.
22. PIKE, R. M. Science, 105 : 391. 1947.
23. RAKOWSKI, P. and LEPPERT, W. Ber. 8 : 788. 1875.
24. THIELE, J. and GUNTHER, F. Ann. Chem. 349 : 45. 1906.

GLYCOSURIA IN PHLORIZINIZED RATS DEPLETED OF PYRIDOXINE[1]

By Edouard Pagé[2] and R. Gingras[3]

Abstract

Glucose excretion and dextrose : nitrogen ratios were measured following phlorizination in young fasting rats depleted of pyridoxine and in their pair-fed controls. The same determinations were also made in older and non-fasting animals following a period of 11 to 16 weeks on a sugar-free, pyridoxine-free ration. Pair-fed rats were again used as controls. No significant differences were found between groups. It is concluded that under the experimental conditions described, pyridoxine insufficiency does not exert a specific effect on gluconeogenesis.

Introduction

The members of the vitamin B_6 group have been shown by Gale and Epps (3) and by Gunsalus and Bellamy (4) to be converted into the coenzyme of amino acid decarboxylases. The importance of these vitamins in transamination reactions has been demonstrated further by Schlenk and Snell (12) and by Lichstein *et al.* (8). In 1941, McHenry and Gavin (9) had already suggested that pyridoxine was essential for the metabolism of protein. Cerecedo and Foy (2) then found that pyridoxine depletion in rats could be accelerated by maintenance of the animals on a high protein diet. This close relationship between pyridoxine and amino acid metabolism suggests that gluconeogenesis might be affected by a lack of this vitamin. D/N ratios were therefore determined in phlorizinized rats that had previously been depleted of pyridoxine. Bartlett and Gaebler (1) have recently reported that the low fasting D/N ratio of a depleted dog could be raised by the administration of pyridoxine. In another instance, desoxypyridoxine seemed to contribute to a low D/N ratio in a dog receiving a sugar-free ration.

Experimental

In a first experiment, young albino rats averaging 53 gm. body weight were put on the following ration: sucrose, 72; "Vitamin Test" casein (Smaco), 18; corn oil (Mazola), 4; Salt Mixture, 4; and Cellu flour, 2 parts. One hundred grams of ration contained: thiamine hydrochloride, 0.4; riboflavin, 0.4; pyridoxine hydrochloride, 0.0 or 0.5; calcium pantothenate, 3.0; nicotinic acid, 3.0; inositol, 10; 2-methyl-1,4-naphthoquinone, 0.1; and choline chloride, 150 μgm. The rats also received two drops weekly of a fish liver oil concentrate enriched with 0.5 μgm. of α-tocopherol per drop. The salt mixture

[1] *Manuscript received February 3, 1948.*
Contribution from the Department of Biochemistry of the Faculty of Medicine, Laval University, Quebec, Que.
[2] *Assistant Professor.*
[3] *Professor of Biochemistry.*

consisted of U.S.P. Salt Mixture XII, No. 2, to which had been added the following: potassium iodide, 0.7; copper sulphate pentahydrate, 0.3; manganese sulphate tetrahydrate, 5.0; and zinc carbonate, 0.2 gm. per kilogram of salt mixture.

Litter mates were paired according to weight and one rat of each pair received the full ration while the other received the pyridoxine-free diet. The food was weighed each day and the consumption of the control animal was limited to that of his mate on the depletion ration. After six weeks, measurements were begun and the last pairs of rats to be tested had been on the above rations for nine weeks. The rats were given subcutaneously 50 μgm. of phlorizin dissolved in 0.5 cc. of sesame oil on the first and second mornings of a two day fast. The urine was collected under light mineral oil in the presence of a preservative and determinations were made on each of the two 24 hr. samples. Glucose was determined by the Shaffer–Hartmann method (6) and nitrogen by micro-Kjeldahl digestion, distillation of the ammonia into boric acid, and titration. Ketone bodies were measured by the Van Slyke gravimetric method (6).

The rats were greatly emaciated at the time measurements were begun and several did not survive the two day fast. Results are given in Table I for the 13 pairs of rats where both members survived. While the data for the other pairs were incomplete, they were similar to those given here.

TABLE I

GLYCOSURIA AND KETONURIA IN PYRIDOXINE DEPLETED RATS AND THEIR PAIR-FED CONTROLS (13 pairs)

	Depleted	Controls
Average length of depletion period (days)	52	
Average final weight at the time of phlorizination (gm.)	109 ± 6*	118 ± 7
Glucose excretion, first 24 hr. (μgm. per 100 gm.)	557 ± 56	400 ± 94
Glucose excretion, second 24 hr. (μgm. per 100 gm.)	284 ± 34	276 ± 52
D/N ratios, second 24 hr.	2.82 ± 0.14	2.67 ± 0.17
Ketone bodies, second 24 hr. (μgm. per 100 gm.)	116 ± 24	71 ± 13

* *Standard deviation of the mean.*

In a second experiment, 20 male rats averaging 190 gm. body weight were used. These rats had previously been on a purified diet containing 30% casein. They were in good health and had made average weekly gains of 30 gm. in the preceding five weeks (since weaning). They were put on a sugar-free ration of the following composition: "Vitamin Test" casein (Smaco), 72; mineral mixture, 4; soybean oil, 20; Cellu flour, 4 parts. One hundred grams of ration contained: thiamine hydrochloride, 0.4; riboflavin, 0.4;

pyridoxine, 1.0; calcium pantothenate, 3.0; nicotinic acid, 3.0; inositol, 10; 2-methyl-1,4-naphthoquinone, 0.1; and choline chloride, 150.0 μgm. The mineral mixture was the same as that used previously and a fish liver oil concentrate fortified with α-tocopherol was also given.

Following three weeks on this regimen, the rats were divided into eight pairs for paired feeding purposes, one rat remaining on the basal ration and the other receiving that ration less its pyridoxine. The remaining four rats were fed *ad libitum*.

After periods of time varying between 77 and 113 days, the D/N ratios were measured on the non-fasting animals. They received each morning a subcutaneous injection of 30 μgm. of phlorizin in sesame oil. Immediately thereafter 24 hr. collections of urine were started. The control rats were injected one day after the depleted ones so that the glucose and nitrogen excretions should correspond to similar food intakes for each rat of a pair. Before any depleted rat was phlorizinized, it was ascertained that a urine sample gave a positive test for xanthurenic acid as evidenced by the appearance of a dark green color when ferric chloride was added to the neutralized urine (7).

Fig. 1 shows the changes in body weight of the three groups from the time the animals were paired until measurements were begun. During the three weeks preceding pairing, the animals had first lost a little weight and then regained it. It is thus seen that the rats fed *ad libitum* remained at an almost stationary weight for some seven weeks before resuming growth.

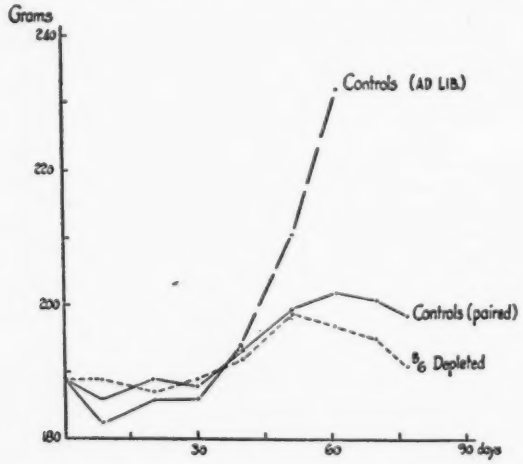

FIG. 1

Table II shows the results of the glucose and nitrogen determinations as well as other pertinent data.

TABLE II

GLYCOSURIA IN PYRIDOXINE DEPLETED RATS AND THEIR PAIR-FED CONTROLS

Pair No.	Depletion period, days	Depleted or controls	Final body weight	Changes in body weight, gm.		D/N ratios		Glucose excretion, μgm.	
						1st day	2nd day	1st day	2nd day
1	77	D	202	−30*	−36**	2.67	1.88	1600	1294
		C	237	1	−5	2.41	2.62	1780	2212
2	77	D	196	0	−30	2.97	3.28	2122	1188
		C	192	−4	−24	2.91	3.08	2020	940
3	84	D	180	−3	−9	2.85	3.20	1546	980
		C	204	21	−7	3.15	3.41	1652	1050
4	84	D	183	12	−1	3.32	3.48	2150	1032
		C	180	10	−3	2.92	3.49	1733	558
5	91	D	195	8	−6	3.34	3.45	1498	918
		C	213	23	0	3.63	3.30	997	1102
6	91	D	142	2	2	2.97	2.15	1652	858
		C	122	−22	−22	2.04	2.36	983	1139
7	113	D	220	14	−22	2.96	2.45	2432	1968
		C	237	33	−4	2.81	2.79	1946	1901
8	113	D	182	−15	−30	3.31	2.70	1865	864
		C	199	7	−29	3.05	2.30	2010	1021
Averages		D	188	−2	−17	3.05	2.82	1858	1138
		C	198	8	12	2.87	2.92	1640	1240

* *From initial body weight.*
** *From highest weight reached.*

Discussion

It has been shown by other workers (2, 10) that rats can survive for weeks and even months on a pyridoxine-free ration and it is difficult to estimate the degree of depletion in this vitamin over relatively short periods of time. One may also question which degree of vitamin insufficiency is best suited for the study of metabolic disturbances resulting directly therefrom. It is clear that should the condition proceed until the onset of severe clinical symptoms, all metabolic functions may be so impaired directly or indirectly as to confuse the issue. In the present experiments, measurements were made at a time when the rats were considered to be moderately deficient in pyridoxine. Paired feeding was deemed essential to distinguish the specific effects of a lack of pyridoxine from indirect ones resulting from general undernutrition or an unsuspected lack of some other factor.

In the first experiment, where young rats were used, signs such as rustiness of the fur and acrodynia were observed in addition to the reduced growth rate. The fact that several rats did not survive a two day fast in a diabetic condition would indicate that the degree of inanition was as advanced as the

subsequent experimental procedure would allow. When older rats were used, it is inferred that they had a large reserve of pyridoxine at the beginning of the depletion period since they had been previously on a high protein, high pyridoxine diet, two conditions favoring a stocking of the vitamin in the tissues (13, 14). It may be seen from Fig. 1 that the lack of pyridoxine did not make itself felt on the body weights for about six weeks, the rats fed *ad libitum* behaving similarly until then. By the time measurements were made, however, all the depleted rats had reached a plateau or were losing weight. Their appearance was much worse than would be deduced from the weight changes and it is likely that they grew in length while losing their fat reserves. They were greatly emaciated and had an unthrifty-looking fur.

The differences in body weight between the depleted rats and their pair-fed controls are not statistically significant.

We are at a loss to explain the sudden resumption of growth of the rats fed *ad libitum* or the similar tendency evidenced by the others at the same time and on a separately prepared ration (Fig. 1).

Widely fluctuating values were found in the first experiment with no significant differences between rats of the same pair except in respect of ketonuria, which was somewhat higher in the depleted animals. The high excretion of glucose by the depleted rats on the first day of fast (Table I) may be due to the fact that these rats ate at leisure during the day and night preceding phlorizination while their mates, which usually ate ravenously upon being fed their quota, had probably been fasting since their last feeding 24 hr. before. It is thought that some of the low D/N ratios may have been due to inanition. Values above 3.0 have been found by us for thriving rats during fast.

In the second experiment, we likewise find no difference in the D/N ratios between depleted and control animals. Several values are rather low, the two lowest recorded being given by the two rats who had lost the most weight since the beginning. One was a depleted rat (Pair 1) and the other was a control rat (Pair 6). Phlorizination for a longer period of time might have revealed some differences between groups. On the other hand, it is interesting to note that although these rats had not eaten any sugar for 11 to 16 weeks, the depleted ones excreted at least as much glucose as their controls on the first day following phlorizination. These initial values may be more indicative of the physiological state of these animals than those that would result from prolonged experimental diabetes when metabolism is of necessity more perturbed.

On the basis of the above findings, it is concluded that pyridoxine does not exert a specific effect on gluconeogenesis. Such a conclusion, however, is difficult to reconcile with the statement that this vitamin is directly concerned in fat synthesis from proteins (9) since it is generally believed that such a synthesis is effected by the glucose (or pyruvic acid) pathway (11, p. 446; 5). One would expect any interference with this process resulting from pyridoxine insufficiency to manifest itself before the amino acids have reached the glucose stage.

Acknowledgments

This work was carried out with the financial assistance of the National Research Council of Canada. The authors wish to express their thanks to Miss M.-R. Lavoie and to Mr. N. Lemay for their valuable technical help.

References

1. Bartlett, P. and Gaebler, O. H. Fed. Proc. 6 : 236. 1947.
2. Cerecedo, L. R. and Foy, J. R. Arch. Biochem. 5 : 207. 1944.
3. Gale, E. F. and Epps, H. M. R. Biochem. J. 38 : 250. 1944.
4. Gunsalus, I. C. and Bellamy, W. D. J. Biol. Chem. 155 : 357. 1944.
5. Gurin, S., Delluva, A. M., and Wilson, D. W. J. Biol. Chem. 171 : 101. 1947.
6. Koch, F. C. and Hanke, M. E. Practical methods in biochemistry. Williams & Wilkins Company, Baltimore. 1943.
7. Lepkovsky, S. and Nielsen, E. J. Biol. Chem. 144 : 135. 1942.
8. Lichstein, H. C., Gunsalus, I. C., and Umbreit, W. W. J. Biol. Chem. 161 : 311. 1945.
9. McHenry, E. W. and Gavin, G. J. Biol. Chem. 138 : 471. 1941.
10. Miller, E. C. and Baumann, C. A. J. Nutrition, 27 : 319. 1944.
11. Peters, J. P. and Van Slyke, D. D. Quantitative clinical chemistry. Interpretations. Vol. 1. Williams & Wilkins Company, Baltimore. 1946.
12. Schlenk, F. and Snell, E. E. J. Biol. Chem. 157 : 425. 1945.
13. Schweigert, B. S., Sauberlich, H. E., Elvehjem, C. A., and Baumann, C. A. J. Biol. Chem. 165 : 187. 1946.
14. Sheppard, E. C. and McHenry, E. W. J. Biol. Chem. 165 : 649. 1946.

THE DIET AND HORMONALLY INDUCED NEPHROSCLEROSIS[1]

E. C. HAY,[2] J. L. PRADO,[3] AND H. SELYE[4]

Abstract

Kidney lesions resembling those of chronic nephritis and nephrosclerosis developed in rats treated with L.A.P. (lyophilized anterior pituitary) and fed 'Purina', but did not develop in similar rats similarly treated and fed Purina mixed with cornstarch in a 1 : 1 ratio. When synthetic diets, differing only in their relative carbohydrate and protein content, were fed, only those L.A.P.-treated rats that consumed a regimen containing 30% casein and 54% cornstarch developed nephrosclerosis. No such lesions were seen in treated rats kept on a 15% casein and 69% cornstarch diet. This difference in response is due to the protein and not to the cornstarch content of these diets; the entire amount of cornstarch was substituted by wheat starch or 15% of it was replaced by an equicaloric amount of fat, without influencing the development of kidney lesions.

Various protein preparations differed in their ability to cause kidney damage. Casein, egg albumen, and wheat gluten were more damaging than lactalbumin, gelatin, or zein.

The severity of nephrosclerosis caused by L.A.P. roughly paralleled the kidney hypertrophy, adrenal enlargement, and increased appetite for water, but not necessarily the food intake or the growth rate. The appearance of these renal lesions was not associated with any gross change in urine pH, but was preceded by a marked albuminuria. By the fifth day, large amounts of albumin were present in the urine of the majority of treated rats consuming the 30% casein diet. Albumin rarely appeared in the urine of treated rats consuming the 15% casein diet.

These findings were discussed in relation to the literature concerning the dietary production of chronic nephritis. The procedure described in this paper greatly accelerates the development of kidney lesions, apparently identical with those shown previously to ensue on the feeding of high protein diets.

Introduction

It has been amply demonstrated that the chronic treatment of rats with desoxycorticosterone acetate (D.C.A.), or crude cattle anterior-pituitary preparations, is followed by the development of kidney lesions similar to those seen in malignant hypertension (45-48). These lesions are characterized by thickening and necrosis of arteriolar walls, enlargement and hyalinization of glomeruli and the formation of hyaline casts with resultant marked tubular dilatation. D.C.A. produces discrete lesions in six to eight weeks in normal rats, but partial nephrectomy and a high salt intake greatly aggravate and accelerate the response (48, 49). Similarly, a high salt intake and partial nephrectomy sensitize the rat to the nephrosclerotic activity of anterior-pituitary preparations (16). Other dietary factors are also of importance (5)

[1] *Manuscript received in original form July 25, 1947, and, as revised, January 23, 1948. Contribution from the Department of Anatomy, McGill University, and l'Institut de Médecine et de Chirurgie expérimentales, Université de Montréal, Montreal, Que.*

[2] *Research Associate, Université de Montréal.*

[3] *Fellow of the Canada-Brazil Trust Fund, from Instituto Butantan, São Paulo, Brazil.*

[4] *Professor and Director of l'Institut de Médicine et de Chirurgie expérimentales, Université de Montréal.*

since rats consuming 'Purina Fox Chow'* and treated with crude anterior-pituitary, develop severe kidney lesions, while similar rats similarly treated but fed 'Pablum'**, remain completely free. In the present communication a report is given of a series of experiments designed to determine to what dietary factor or factors this marked difference in response could be attributed.

Experimental

The reported composition of 'Purina' differs greatly from that of Pablum both in the types and in the proportions of food materials used in its preparation. For example, there is a marked difference in the relative amounts of carbohydrate and protein present. Purina contains approximately 50% carbohydrate and 26% protein, while Pablum contains about 70 and 15%, respectively. By adding cornstarch to Purina it is possible to prepare a mixture of approximately the same carbohydrate–protein ratio as Pablum. The first experiment was performed to determine whether such a change in the Purina diet would influence the nephrosclerotic activity of lyophilized beef anterior-pituitary (L.A.P.)***.

The Nephrosclerotic Activity of L.A.P. in Rats Consuming a Modified Purina Diet

Procedure: Four groups of 10 male piebald rats, weighing 40 to 60 gm. (average 51 gm.), were castrated, partially nephrectomized, and given 1% sodium chloride to drink. One group was fed ground Purina, a second, 50% Purina, and 50% cornstarch. The two remaining groups were fed these diets and were injected subcutaneously twice daily with 10 mgm. of L.A.P. The diets were prepared daily by mixing with enough water to form firm cakes. After 26 days of treatment, all surviving rats were sacrificed.

At autopsy, the kidneys and adrenals were fixed in 'Suza' for 24 hr., then transferred to 4% formalin. They were then dissected and weighed on an analytical balance; kidneys were sectioned for histological study. The kidney weights were determined in order to establish whether the diet affected them in the same manner as it did the development of renal lesions. The adrenals were weighed because adrenalectomy inhibits the nephrosclerotic activity of L.A.P. (9) and because it has been claimed by some and refuted by others that the proportion of protein in the diet influences their weight (4, 18, 21, 50, 55). The incidence and severity of nephrosclerosis was determined microscopically employing a scale ranging from 0, to indicate no lesions, to +++, to represent maximal damage.

** A commercial animal food prepared by Ralston Purina Co. Ltd., Montreal, Que.*

*** A commercial infant food prepared by Mead Johnson and Company of Canada, Limited, Belleville, Ont.*

**** L.A.P. is prepared as follows: whole cattle pituitaries are removed from the skulls at the abattoir and immediately frozen in dry ice. At the laboratory, the glands are thawed, freed of posterior lobe and connective tissue, minced, and refrozen in bulbs. The bulbs are dehydrated in a high vacuum system containing a trap cooled with dry ice. The dry powder obtained suspends easily in water or 10% alcohol and passes through a No. 20 hypodermic needle.*

Results: In Table I, the data obtained in Expt. 1 are summarized. Kidney weights are expressed in terms of mgm. per 100 cm.^{2} of body surface area. The incidence and severity of renal lesions are shown as percentages of the maximal ever observed.

TABLE I

INFLUENCE OF DIET ON THE NEPHROSCLEROTIC ACTIVITY OF L.A.P.

(Averages and standard errors)

Group	Treatment		No. of rats	Final body weight, gm.	Kidney			Adrenal, mgm.
	Dietary	Hormonal			Mgm. per 100 cm.^{2} body surface area	Nephrosclerosis		
						Incidence	Severity	
1	Purina	—	10	104 ± 5	418 ± 7	0	0	27 ± 2
2	50% Purina, 50% starch	—	9	93 ± 3	319 ± 10	0	0	25 ± 2
3	Purina	L.A.P.	8	131 ± 6	686 ± 32	88	38	64 ± 2
4	50% Purina, 50% starch	L.A.P.	5	140 ± 7	425 ± 6	0	0	41 ± 2

First, it should be pointed out that the 50% Purina diet was not markedly deficient in food essentials since the body growth rates of treated or untreated rats consuming this diet did not differ significantly from those of rats eating 100% Purina. No significant difference was seen in the adrenal weights of untreated rats consuming the two diets. However, the relative kidney weights of animals given 100% Purina (Group 1) were much greater than those of rats given 50% Purina (Group 2). No renal lesions were observed in any of these rats. With L.A.P. treatment (Groups 3 and 4), adrenal and kidney weights were increased in both groups, but much more in the group fed Purina only. Nephrosclerosis occurred in seven of the eight surviving animals given the 100% Purina diet, and in none of those eating the Purina–starch mixture.

These observations show that the diet can profoundly influence the development of the kidney lesions induced by hormone treatment. It is not clear, however, to what dietary factor the observed effects were due, since adding starch to the Purina not only increases the carbohydrate and decreases the protein content of the diet, but also decreases the proportion of fat and vitamins. It was decided to continue this study using simple synthetic diets whose constituents could be so varied that only two food factors would be changed at one time.

NEPHROSCLEROTIC ACTIVITY OF L.A.P. IN RATS ON TWO BASIC SYNTHETIC DIETS. THE COMPOSITION OF THE TWO BASIC SYNTHETIC DIETS

The constituents of the two basic synthetic diets and their proportions are given in Table II. The proportion of protein to carbohydrate in Diet 2 was chosen to coincide with that reported for Pablum, while the proportion in Diet 1, was made somewhat greater than that reported for Purina.

TABLE II

COMPOSITION OF TWO BASIC SYNTHETIC DIETS

	Diet No.	
	1	2
Composition, %		
Cornstarch	54	69
Casein*	30	15
Fat**	1	1
Cod liver oil ***	1	1
Bulk†	1	1
Sodium chloride ††	4	4
Mineral mixture‡	4	4
Water †††	5	5
Supplements, mgm. per 100 gm. of diets		
Thiamine hydrochloride‡‡	0.4	
Riboflavin‡‡	0.4	
Pyridoxin‡‡	0.4	
Calcium pantothenate‡‡	2.0	
Nicotinic acid‡‡	1.0	
α-Tocopherol acetate‡‡	About 10 mgm. per rat once weekly introduced by dropper into mouth	

* *Not vitamin-free. It was considered that this would supply the choline requirement.*

** *Crisco, Domestic Shortening, or Primex.*

*** *Ingram and Bell.*

† *Agar agar, acacia, or "Cellu-flour". When agar became unavailable we used acacia until Cellu-flour could be obtained. No difference was seen between them.*

†† *This was added to the diet to sensitize the rats to the nephrosclerotic activity of L.A.P. instead of giving it in the drinking water.*

††† *The starch and casein in the first lot of diets was found to contain an amount of water corresponding to 5% of the diet. On the dry weight basis these diets contain 31.6 and 15.8% protein, respectively. Subsequently the water content of the starch and casein was determined and the amount weighed out calculated to give 54% or 69% starch and 30% or 15% casein by dry weight.*

‡ *Slightly modified mixture recommended by Steenbock and Nelson (52).*

$NaCl$	23.4 gm.
$MgSO_4 . 7H_2O$	24.6 gm.
Na_2HPO_4	14.2 gm.
K_2HPO_4	69.6 gm.
$CaHPO_4 . 2H_2O$	69.8 gm.
Ca-lactate . $5H_2O$	15.4 gm.
Fe-citrate	1.2 gm.
KI	0.16 gm.

‡‡ *Kindly supplied by Hoffman-LaRoche.*

It will be noted that inositol and liver extract are not included in these diets. The effect of adding these has not been investigated, but at the time it was felt that in such short term experiments as those to be described, they

would not be essential. Similarly, a simple salt mixture was employed in preference to a more complete one, since in three weeks, it was unlikely that trace elements would play any role.

The Adequacy of the Two Basic Synthetic Diets

The adequacy of these diets for growth and maintenance has been tested in four different experiments. The data obtained are summarized in Table III.

TABLE III

Growth rates and organ weights with synthetic diets

(Averages and standard errors)

Experiment No.	Group No.	Diet No.	No. of rats	Body weight		Organ weights	
				Initial	Final	Adrenal, mgm.	Kidney, mgm. per 100 sq. cm. body surface
2	1	2	6	46	82 ± 4	19 ± 1	523 ± 9
(W)	2	Pablum	6	46	84 ± 4	17 ± 1	467 ± 12
3	3	2	10	50	98 ± 2	13 ± 2	379 ± 13
(BW)	4	1	10	50	113 ± 3	15 ± 2	454 ± 14
4	5	2A	6	50	106 ± 3	22 ± 1	427 ± 9
(W)	6	1A	6	50	139 ± 5	35 ± 2	595 ± 17
5	7	2A	8	122	251 ± 12	33 ± 2	491 ± 11
(BW)	8	1A	8	125	259 ± 21	30 ± 1	525 ± 19

The means given for Expts. 3 and 4 are also the untreated control values for the treated animals in the experiments to be described below. Different strains of rats were employed in different tests. The strain used is indicated by letters placed below the experiment number: *W*, for white (albino), and *BW*, for black-and-white or piebald. This factor is of importance when comparisons are made between experiments. Both strains respond to the same stimuli although average organ weights differ somewhat.

In *Expt. 2*, the body growth rate and organ weights of immature male rats eating Diet 2 for 21 days were compared with those of similar rats consuming Pablum. No difference in growth rate was seen. Of the organs weighed, only the relative kidney weights were significantly different. The cause of this is unknown; it might be due to a difference in the type of protein eaten.

In *Expt. 3*, a comparison was made of the body growth rates and organ weights of immature castrate rats partially nephrectomized and fed Diets 1 and 2 for four weeks. The growth rate of those fed Diet 2 was slightly less

than that of the rats eating Diet 1. No difference in organ weights was noted except in relative kidney weights. As would be expected, those animals consuming the low protein and high carbohydrate diet had the smaller kidneys.

Both these groups were in excellent condition, but in L.A.P. treated rats, Diet 2, unlike Purina, Pablum, or Diet 1, was quite inadequate for maintenance and rapid growth. Calculation suggested that the daily vitamin B intake was probably just adequate under normal conditions, but not after stimulation of metabolism with pituitary hormones. In experiments performed after this time, the vitamin B content of both diets was doubled. In addition, 100 mgm. of choline chloride was added per 100 gm. of the two diets. This markedly improved the condition of treated rats consuming the low protein, high carbohydrate diet, but had no apparent effect on that of the animals consuming Diet 1.

In *Expt. 4*, a comparison was made of the growth rates and organ weights of two groups of six immature, castrate rats, partially nephrectomized and eating these two diets so supplemented (Diets 2A and 1A, Groups 5 and 6). The additional vitamins markedly improved the growth rate. As might be expected, the animals on the low protein diet grew less rapidly, perhaps because their methionine intake was slightly below that considered essential for rapid growth (41). The adrenal and relative kidney weights of rats on Diet 1A were significantly larger than those of rats consuming Diet 2A.

Expt. 5 showed that with somewhat older rats, these two diets (Diets 2A and 1A, Groups 7 and 8), sufficed to maintain normal growth and good health for more than 11 weeks. The absence of any difference in growth rate further indicated that the inhibition noted in very young rats could be due to lack of methionine. As before, only the kidney weights differed significantly in the two groups.

It appears that the two basic synthetic diets were adequate for the normal growth of rats weighing 120 gm. or more; rats weighing 40 to 60 gm. grew less well on Diet 2 than on Diet 1 or Pablum.

The Nephrosclerotic Activity of L.A.P. in Rats Consuming Synthetic Diets

The nephrosclerotic activity of L.A.P. in rats kept on the above-mentioned diets was determined employing a previously described assay method (16). Male rats weighing 40 to 60 gm. were castrated, partially nephrectomized, and subcutaneously injected with L.A.P. for 21 days commencing one day postoperatively. In *Expt. 6*, the animals received 25 mgm. and in the rest, 20 mgm. daily, in two 0.3 cc. portions of a 10% alcoholic suspension. The average amount of dry food and water consumed by each rat was determined for the last five days in Expts. 6 to 9. As before, the fixed adrenals and kidneys were weighed, and the incidence and severity of nephrosclerosis were diagnosed histologically. Results of Expts. 6 to 9 are tabulated in Table IV

TABLE IV

THE EFFECT OF THE PROPORTION OF PROTEIN IN THE DIET CONSUMED ON THE NEPHROSCLEROTIC ACTIVITY OF L.A.P.

(Averages and standard errors)

Experiment No.	Group No.	Diet		No. rats	Final body weight, gm.	Nephrosclerosis		Organ weights		Food intake, gm./rat/day	Water intake, cc./rat/day
		No.*	Modification			Incidence	Severity	Adrenal, mgm.	Kidneys, mgm./100 cm.² body surface		
6	1	2		4	122 ± 7	0	0	48 ± 4	516 ± 17	10.7 ± 0.6	37 ± 2
(BW)	2	1		10	163 ± 5	90	47	71 ± 5	858 ± 50	9.0 ± 0.4	49 ± 4
	3	1	Casein 50% Starch 34%	8	164 ± 10	100	61	81 ± 7	1107 ± 43	9.2 ± 0.6	99 ± 9
	4	2	Double B-vitamins	9	115 ± 5	0	0	53 ± 4	534 ± 15	7.2 ± 0.5	32 ± 2
	5	2	Choline added	8	132 ± 5	0	0	49 ± 2	536 ± 20	9.3 ± 0.3	38 ± 2
7	1	2		5	113 ± 9	0	0	58 ± 2	650 ± 36	13 ± 0.7	28 ± 3
(BW)	2	2	Double B-vitamins** Choline added	10	122 ± 4	0	0	58 ± 3	540 ± 19	13 ± 0.7	33 ± 2
	3	1		5	146 ± 13	100	93	78 ± 7	983 ± 62	17 ± 1.2	58 ± 1
	4	1	Double B-vitamins Choline added	8	151 ± 5	100	93	80 ± 4	991 ± 48	16 ± 0.7	57 ± 2
8	1	1A		4	151 ± 8	100	92	91 ± 5	968 ± 84	17 ± 1	67 ± 4
(BW)	2	2A	Cornstarch replaced by wheat starch	3	104 ± 10	0	0	46 ± 4	472 ± 33	13 ± 1	23 ± 2
	3	1A	Cornstarch replaced by wheat starch	7	172 ± 5	86	53	72 ± 3	763 ± 66	17 ± 1	38 ± 3
	4	2A	Fat 8.5% Cornstarch 54%	8	115 ± 7	0	0	41 ± 2	458 ± 23	11 ± 1	30 ± 2
9	1	2A	Fat 8.5%	10	140 ± 6	0	0	54 ± 3	564 ± 23	16 ± 1	39 ± 3
(W)	2	2A	Cornstarch 54%	7	133 ± 3	0	0	57 ± 4	613 ± 39	15 ± 1	38 ± 3
	3	1A		9	148 ± 5	90	74	64 ± 3	1101 ± 74	15 ± 1	52 ± 4
	4	1A	Fat 8.5% Cornstarch 39%	9	154 ± 8	100	80	78 ± 5	1101 ± 52	14 ± 1	59 ± 5

* *See Table II.*

** *Subsequently designated by "A."*

and of Expts. 10 and 11 in Table V. They are expressed in the same terms as were employed in Table I. All groups originally consisted of 10 rats; the number indicated in the table is the number surviving to autopsy. Average initial body weights are omitted since they ranged from 52 to 54 gm. throughout.

TABLE V

THE INFLUENCE OF THE TYPE OF PROTEIN IN THE DIET ON THE NEPHROSCLEROTIC ACTIVITY OF L.A.P.

(Averages and standard errors)

Experiment No.	Group No.	Diet		No. rats	Final body weight, gm.	Nephrosclerosis		Organ weights	
		No.	Modification			Incidence	Severity	Adrenal, mgm.	Kidneys, mgm./100 cm.^{2} body surface
10	1	1A		6	178 ± 9	100	82	63 ± 4	1100 ± 74
(W)	2	1A	Casein 15% / Gelatin 15%	5	121 ± 7	80	47	54 ± 1	963 ± 16
	3	1A	Casein 15% / Zein 15%	9	152 ± 6	78	44	62 ± 4	796 ± 41
	4	1A	Casein 15% / Egg albumen 15%	8	144 ± 14	100	83	68 ± 3	1122 ± 53
	5	1A	Casein 15% / Gluten 15%	7	157 ± 5	100	71	65 ± 4	1079 ± 84
11	1	1A		9	169 ± 6	100	80	69 ± 4	1066 ± 50
(W)	2	1A	Casein 15% / Gelatin 15%	10	135 ± 5	70	30	53 ± 2	906 ± 48
	3	1A	Casein 15% / Zein 15%	10	154 ± 6	70	40	56 ± 2	649 ± 43
	4	1A	Casein 15% / Egg albumen 15%	6	137 ± 8	83	67	62 ± 5	1002 ± 83
	5	1A	Casein 15% / Lactalbumin 15%	9	151 ± 5	66	54	59 ± 2	789 ± 52
	6	1A	Casein 0% / Lactalbumin 30%	9	150 ± 7	56	26	56 ± 3	588 ± 16

The dietary factors studied included the effects of: (1) varying the ratio of protein to carbohydrate, (2) increasing the vitamin B content, (3) substituting wheat starch for cornstarch, (4) replacing a portion of the starch with an equicaloric amount of fat, (5) changing the source of protein.

(1) *Effect of Varying the Ratio of Protein to Carbohydrate and* (2) *of Increasing the Vitamin B Content of the Diet*

In *Expt. 6*, the nephrosclerotic effect of L.A.P. was assayed in rats consuming five different diets. Those in Group 1 were fed basic diet No. 2 (15% casein), and those in Group 2, basic diet No. 1 (30% casein). Rats in Group 3

consumed a diet in which starch was replaced by casein to make the regimen contain 50% casein. Animals in Group 4 were given Diet 2 supplemented with twice the amounts of B vitamins indicated in Table II. Those in Group 5 were fed Diet 2 supplemented with 100 mgm. of choline chloride per 100 gm. of diet. Nephrosclerosis developed only in those rats consuming diets containing 30% or more of casein. No kidney lesions were observed in rats eating any of the three diets containing 15% casein. The development of kidney lesions showed no relationship to the average food intake during the last five days, but was roughly proportional to the increase in body weight, adrenal size, relative kidney weight, and water intake. Increasing the concentration of B vitamins and choline decreased the mortality and greatly improved the condition of rats fed the low protein diet.

In *Expt. 7*, we further investigated the effect of vitamin B and choline chloride supplements on survival and the development of kidney damage. Four groups were employed, those in the first were given Diet 2; the second, Diet 2 modified (Diet 2A); the third, Diet 1; and the fourth, Diet 1 similarly modified (Diet 1A). Survival was again improved by the extra addition of these vitamins. Consequently they were added in all subsequent experiments. The addition of these vitamins also decreased the kidney weight of animals eating the low protein diet, but had no significant effect on adrenal enlargement or the development of kidney lesions. Again a rapid growth rate, large adrenals, large kidneys, and greatly increased water intake were associated with the occurrence of nephrosclerosis. In this experiment the rats consuming the low protein, high carbohydrate diet ate less in the last five days, than those given the high protein, low carbohydrate regimen, hence their salt intake was not the same. However, the difference involved could not be responsible for the difference in their sensitivity to the development of nephrosclerosis, since in Expt. 9 the difference in food intake was not manifest while that in the occurrence of kidney lesions was.

(3) *Effect of Substituting Wheat Starch, or* (4) *Fat, for Cornstarch*

It was possible that the nephrosclerosis preventing effect of the 69% cornstarch diet was due specifically to this starch. To investigate this, in *Expt. 8*, the whole of the cornstarch was substituted by wheat starch. To determine whether fat was also effective, a portion of starch in Diet 2A was replaced with an equicaloric amount of vegetable fat (Crisco) so that the diet contained the same proportion of starch as the 30% casein basic diet (Diet 1A). The difference in the weight of starch removed and fat added was made up by adding bulk (agar agar).

Nephrosclerosis occurred in rats eating the 30% casein, 54% wheat starch diet (Group 3) just as it did in those consuming the 30% casein, 54% cornstarch regimen (Group 1). No kidney lesions were seen in rats eating either the 15% casein, 69% wheat starch diet (Group 2), or the 15% casein, 54%

cornstarch, and 8.5% fat regimen (Group 4). Although survival was low in the first two groups owing to intercurrent infections, the results were so conclusive that it was decided not to repeat them.

However, the ability of fat to effect the same protective action as carbohydrate in the low casein diet was confirmed in *Expt. 9*. No kidney lesions appeared in rats eating food composed either of 15% casein, 69% starch, and 2% fat; or of 15% casein, 54% starch, and 8.5% fat. The development of severe damage likewise failed to be influenced by substituting fat for a similar amount of starch in the diet containing 30% casein and 54% starch.

In both these experiments, as in the previous two, the development of nephrosclerosis was associated with a rapid growth rate, markedly enlarged adrenals and kidneys, and a greatly increased water intake.

Thus, since wheat starch, vegetable fat, and probably other carbohydrates and fats can replace cornstarch, the kidney damaging effect of the 30% casein diet must have been due to the presence of protein and not to the lack of carbohydrate.

(5) *Effect of Substituting Various Proteins for Casein*

In Expts. 2 to 9, casein was used as the source of protein. To determine whether all proteins are equally toxic we examined the effect of substituting half of the casein in the 30% casein diet with the following protein preparations: gelatin, lactalbumin, zein, egg albumen, and gluten. Table V summarizes the results of Expt. 10, in which gelatin, zein, egg albumen, and gluten were used. It will be seen that the incidence and severity of kidney lesions differed greatly in the various groups. These differences are larger than could be expected on the basis of chance since the standard error of the incidence of the response in the four groups of rats consuming Diet 1A, unmodified, in Expts. 8 to 11 is 3 and that of the severity is 4. As in previous experiments the severity of kidney damage is correlated with the increase in adrenal and kidney weights.

Expt. 11 was essentially a repetition of the previous one with the addition of Groups 5 and 6 in which lactalbumin replaced respectively half or the total casein. From the results shown in Table V, it is seen that there was remarkable agreement with the responses obtained in the previous experiment, in respect to the incidence and severity of kidney lesions, growth rate, and the increase in adrenal and kidney weights.

These experiments show, therefore, that in rats injected with crude anterior-pituitary, the consumption of casein, egg albumen, and wheat gluten is associated with the development of greater kidney damage than is seen when lactalbumin, zein, and gelatin are eaten. We are now investigating whether the difference between these proteins is due to their different amino acid composition.

Discussion

Previous Observations

Diet and Chronic Nephritis

The influence of the protein content of the diet on the development of nephritis, nephrosclerosis, and malignant hypertension, has long been a subject of controversy. Workers who obtained kidney damage by giving protein-rich diets considered the lesions to be similar to those seen in chronic nephritis (13, 30, 33, 36, 43, 57). Workers who did not obtain lesions after prolonged treatment, claimed inadequate or unnatural diets were responsible for the positive results (2, 20, 35).

Older animals were found more responsive than young (19, 27, 51) and chronic nephritis occurs spontaneously in old rats and in rabbits of any age, hence it was suggested that the incidence of lesions obtained in the earlier experiments was not significant (35). More recently, partial nephrectomy (19, 27, 7), the use of liver as the source of protein, and treatment with thyroxin have reduced the required duration to three or four months (6). The lesions obtained in this shorter period were very similar to those seen normally in old rats.

The ability of various amino acids to reproduce the kidney-damaging effect of proteins has been investigated by several authors. Although tubular damage was noted after injecting or feeding a number of amino acids (12, 17, 22, 28, 32, 53), glomerular or arteriolar injury was mentioned only by Newburgh and Marsh (32) who reported prolonged albuminuria with the toxic amino acids, and Hueper and Martin (17), who observed hyalinization of the media of arterioles with tyrosine. Marked kidney enlargement followed the addition of glutamic acid to the diet but no specific lesions were noted (23). A probable metabolite of glutamic acid, glutaric acid, has been reported to be both toxic and nontoxic (39, 40, 58). Rose (40) obtained tubular nephritis with and without glomerular involvement, when the sodium salt of this acid was injected. In no case, however, did the damage approximate that seen with long protein feeding. Thus the effect of a high protein intake is probably not due merely to the absorption of large amounts of any one amino acid. It could be due, however, to an imbalance between the supply of certain amino acids and the rate of their utilization or destruction. Supporting such a suggestion of an indirect effect is the fact that the administration of certain amino acids or high protein diets is known to influence vitamin requirements (3, 6, 8, 11, 26, 29, 37, 38, 42, 44, 56).

Diet and Renal Hypertrophy

The effect of dietary protein on kidney enlargement has also been a subject of controversy. Those authors who observed kidney lesions described marked kidney enlargement. A similar renal hypertrophy was also seen by workers who found no lesions that they considered characteristic of chronic nephritis

(2, 34, 35). Since excessive diuresis, tubular dilatation, and damage were noted, it is possible that nephrosclerosis would have developed after a longer period of treatment. In short term experiments, MacKay *et al.* (25) noted that the amount of enlargement very closely paralleled the casein intake. Subsequently it was found (24) that the hypertrophy is greatly lessened, but not entirely prevented, if the same yeast extract: casein ratio is maintained in the low and high protein diets.

Diet and Adrenal Hypertrophy

Significant adrenal enlargement was noted in animals fed protein-rich diets by Tepperman *et al.* (55), but not by Ingle *et al.* (18) or others (4, 21). It is possible that the vitamin intake might play a role in enlargement as it certainly does in the maintenance of normal adrenal structure (29, 54).

Chronic Nephritis and the Growth Rate

It has been remarked repeatedly that chronic nephritis occurs more readily in otherwise healthy and well-developed animals than in the ill-fed. For example, Newburgh and Curtis (30) pointed out that "the diets that caused the most injury also permitted the best growth". Similarly, Goldblatt *et al.* (14) stated that a high meat diet induced hypertension only in growing animals. Clinically, when nephritis follows an infection, it tends to occur during convalescence. In pregnancy, it is often seen when the fetus is gaining weight most rapidly. The role of protein in the induction of chronic nephritis has usually been attributed to improper catabolism, but it should be kept in mind that it might be correlated with an anabolic imbalance.

Chronic Nephritis and Caloric Intake

It is also possible that the damage seen is associated with a high caloric intake rather than rapid growth or repair, since in all the above-cited situations, excessive amounts of food are consumed. In support of this possibility is the sensitizing effect of thyroxin. Animals treated with this hormone have greatly increased appetites but may gain or lose weight depending on the adequacy of the diet (10). The finding of Saxton and Kimball (43) that restriction of the food intake reduces the incidence of spontaneous "chronic nephrosis" is also pertinent.

Present Observations

Relation between Chronic Nephritis and Nephrosclerosis

The kidney lesions that we obtained in rats treated with anterior-pituitary preparations are very similar to those shown in the excellent illustrations of Jackson and Moore (19). As with lesions of purely dietary origin, their development is promoted by partial nephrectomy (16), thyroxin (47), and, as shown here, by the feeding of a moderately high protein diet. The effect of a high sodium chloride intake, which is also sensitizing in L.A.P.-treated

rats (16), has not been adequately investigated in respect to the production of purely dietary chronic nephritis. When salt comprised 18 to 25% of the diet, no sensitizing effect was seen (35), but it is probable that the test rats refused to eat adequate amounts of such a diet.

The production of a high incidence and severity of kidney lesions in one-month-old rats treated for 21 days, represents a considerable acceleration of a process that could be expected to run its course in three to five years in a normal rat, about one and one-half years in a partially nephrectomized rat on a protein-rich diet, and three to four months in a partially nephrectomized rat, kept on a similar diet and given thyroid.

Dietary Protein: Carbohydrate Ratio and Nephrosclerosis

There can be no doubt that the production of these kidney lesions with L.A.P. treatment is partly dependent upon the protein intake. Various protein preparations caused different degrees of kidney damage in agreement with the findings of earlier workers who used purely dietary measures to produce renal lesions (13, 30, 31, 43). The feeding of carbohydrate or fat instead of protein protected rats against the nephrosclerosis-producing effect of L.A.P. The growth rate and food intake were often less with the non-damaging synthetic diets: this may have played some role but could not have been the sole cause of the difference in toxicity. As shown in Table I, the growth rate of the animals given the two diets did not differ significantly, yet only those eating Purina developed lesions. Moreover, as shown in Table V, synthetic diets containing different protein preparations permitted identical growth rates with very different degrees of kidney damage.

Vitamin Supply and Nephrosclerosis

The nephrosclerosis is not prevented by a normal supply of vitamins. The same concentration of vitamins was present in the diets of the rats that had pathologic kidneys and those that did not. Further work with vitamins of the B complex is being done, however, in view of the repeated claims that the vitamin intake markedly influences the development of chronic nephritis of purely dietary origin.

Urine pH and Nephrosclerosis

The production of nephrosclerosis in these experiments was accompanied by no gross change in the pH of bladder urine*. The urine pH of untreated castrates kept on Diets 1A and 2A was 6.2 ± 0.1 and 6.2 ± 0.1, respectively (50 determinations each), and that of L.A.P.-treated castrates, 5.9 ± 0.1 and 5.8 ± 0.1, respectively (100 determinations each). It appears that L.A.P. caused no significant decrease in urine pH.

* *Measured visually by expressing bladder urine on two or three ranges of "Accutint" pH papers.*

Urine Albumin and Nephrosclerosis

The urine was tested for albumin in a number of the experiments*. At the end of an experiment, the albumin concentration in the urine of rats kept on Diet 2, modified and unmodified, was the same as in stock rats, while all those receiving Diets 1 or 1A had a moderate to excessive albuminuria. In the latter groups, the increased output began after the fifth day, but was approximately maximal by the 10th day. The amount lost varied somewhat from day to day and with the protein preparation fed. For example, animals consuming the mildly toxic 30% lactalbumin diet averaged on three occasions in the last 10 days an incidence and severity of 66 and 23% albuminuria as compared with 100 and 52% for rats given the highly toxic 30% casein regimen.

Renal Hypertrophy and Nephrosclerosis

In L.A.P.-treated rats the kidney enlargement was roughly proportional to the degree of nephrosclerosis produced. No consistent renal enlargement was seen in groups that developed no lesions; the differences in kidney weight are so small that it is necessary to have untreated control animals with each experiment to demonstrate the purely renotrophic effect of anterior-pituitary preparations (15).

Diet and Adrenal Hypertrophy

The adrenal enlargement also roughly paralleled the development of the kidney lesions, but a very marked increase in adrenal weight occurred also in L.A.P.-treated rats that developed no renal lesions. Occasionally, partial or nearly complete cortical necrosis was seen with excessive adrenal enlargement in L.A.P.-treated animals on Diet 1 modified or unmodified. Adrenal necrosis was not seen in treated animals consuming Diet 2 modified or unmodified.

Acknowledgments

This work was supported by the U.S. Public Health Service and the Commonwealth Fund of New York.

The authors are also indebted to Miss Paulette Séguin for her invaluable technical assistance; Dr. J. S. L. Browne, Royal Victoria Hospital, Montreal, Que., for his interested co-operation; Dr. K. A. Clendenning, National Research Council, Ottawa, for highly purified samples of several starches; Dr. W. E. Parker and Dr. Enid P. Knight, Ogilvie Flour Mills Co. Ltd., Montreal, for wheat gluten; Ayerst, McKenna, and Harrison, Montreal, for protein nitrogen determinations; Dr. Paul Blanc, Hoffman-LaRoche, for generous supplies of B-vitamins and α-tocopherol; and Dr. H. Jensen, DesBergers Laboratories, Montreal, for the anterior-pituitary preparation.

** Determined in expressed urine by adding 0.3 cc. of Esbach's picric and citric acid reagent to 0.3 cc. of urine in a conical 10 cc. centrifuge tube, centrifuging 10 min. at 1500 r.p.m. The incidence and severity of albuminuria, read using a scale of 0 to +++, was then expressed as a percentage of the maximal possible.*

References

1. ADDIS, T., LEE, D. D., LEW, W., and POO, L. J. J. Nutrition, 19 : 199. 1940.
2. ADDIS, T., MACKAY, E. M., and MACKAY, L. L. J. Biol. Chem. 71 : 139. 1926.
3. BASINSKI, R. R. H., and SEALOCK, R. R. Federation Proc. 5 : 121. 1946.
4. BENUA, R. S. and HOWARD, E. Endocrinology, 36 : 170. 1945.
5. BERMAN, D., HAY, E. C., and SELYE, H. Proc. Can. Physiol. Soc. 20. 1945.
6. BLATHERWICK, N. R. and MEDLAR, E. M. Arch. Intern. Med. 59 : 572. 1937.
7. BLATHERWICK, N. R., MEDLAR, E. M., CONNOLLY, J. M., and BRADSHAW, P. J. J. Biol. Chem. 92 : 84 (Proc.). 1931.
8. CERECEDO, L. R. Federation Proc. 5 : 229. 1946.
9. DONTIGNY, P., BÉLAND, E., HALL, E., and SELYE, H. Rev. Can. Biol. 5 : 356. 1946.
10. DRILL, V. A. Proc. Soc. Exptl. Biol. Med. 39 : 313. 1938.
11. DYE, M., BATEMAN, I., and PORTER, T. J. Nutrition, 29 : 341. 1945.
12. EARLE, D. P., JR., SMULL, K., and VICTOR, J. J. Exptl. Med. 76 : 317. 1942.
13. EVANS, N. and RISLEY, E. H. Calif. West. Med. 23 : 437. 1925.
14. GOLDBLATT, H., KAHN, J. R., and LEWIS, H. A. J. Am. Med. Assoc. 119 : 1192. 1942.
15. HAY, E. C. J. Pharmacol. 88 : 208. 1946.
16. HAY, E. C. and SEGUIN, P. Am. J. Physiol. 147 : 299. 1946.
17. HUEPER, W. C. and MARTIN, G. J. Arch. Path. 35 : 685. 1943.
18. INGLE, D. J., GINTHER, G. B., and NEZAMIS, J. Endocrinology, 32 : 410. 1943.
19. JACKSON, H. and MOORE, O. J. J. Clin. Investigation, 5 : 415. 1928.
20. JACKSON, H., JR. and RIGGS, M. D. J. Biol. Chem. 67 : 101. 1926.
21. LEATHEM, J. H. Endocrinology, 37 : 157. 1945.
22. LILLIE, R. D. U.S. Pub. Health Repts. 47 : 83. 1932.
23. MACKAY, E. M. J. Nutrition, 6 : 157. 1933.
24. MACKAY, E. M. Am. J. Physiol. 106 : 571. 1933.
25. MACKAY, E. M., MACKAY, L. L., and ADDIS, T. Am. J. Physiol. 86 : 466. 1928.
26. MILLER, E. C. and BAUMANN, C. A. J. Biol. Chem. 157 : 551. 1945.
27. MOISE, T. S. and SMITH, A. H. Arch. Path. 4 : 530. 1927.
28. MOREHEAD, R. P., FISHMAN, W. H., and ARTOM, C. Am. J. Path. 22 : 385. 1946.
29. MORGAN, A. F., GROODY, M., and AXELROD, H. E. Federation Proc. 5 : 236. 1946.
30. NEWBURGH, L. H. and CURTIS, A. C. Proc. Soc. Exptl. Biol. Med. 24 : 963. 1927.
31. NEWBURGH, L. H. and CURTIS, A. C. Arch. Intern. Med. 42 : 801. 1928.
32. NEWBURGH, L. H. and MARSH, P. L. Arch. Intern. Med. 36 : 682. 1925.
33. NUZUM, F. R. Arch. Intern. Med. 40 : 364. 1927.
34. OSBORNE, T. B., MENDEL, L. B., PARK, E. A., and WINTERNITZ, M. C. Am. J. Physiol. 72 : 222. 1925.
35. OSBORNE, T. B., MENDEL, L. B., PARK, E. A., and WINTERNITZ, M. C. J. Biol. Chem. 71 : 317. 1927.
36. POLVOGT, L. M., MCCOLLUM, E. V., and SIMMONDS, N. Bull. Johns Hopkins Hosp. 34 : 168. 1923.
37. READER, V. and DRUMMOND, J. C. Biochem. J. 20 : 1256. 1926.
38. RICHARDSON, L. R., HOGAN, A. G., LONG, B., and ITSCHNER, K. I. Proc. Soc. Exptl. Biol. Med. 46 : 530. 1941.
39. RINGER, A. I. J. Biol. Chem. 12 : 223. 1912.
40. ROSE, W. C. J. Pharmacol. 24 : 147. 1924.
41. ROSE, W. C. Science, 86 : 298. 1937.
42. SARETT, H. P., KLEIN, J. R., and PERLZWEIG, W. A. J. Nutrition, 24 : 295. 1942.
43. SAXTON, J. A., JR. and KIMBALL, G. C. Arch. Path. 32 : 951. 1941.
44. SELYE, H. Z. ges. exptl. Med. 74 : 320. 1930.
45. SELYE, H. Can. Med. Assoc. J. 47 : 515. 1942.
46. SELYE, H., BÉLAND, E., and STONE, H. Rev. Can. Biol. 4 : 120. 1945.
47. SELYE, H. and HALL, C. E. Arch. Path. 36 : 19. 1943
48. SELYE, H., HALL, C. E., and ROWLEY, E. M. Can. Med. Assoc. J. 49 : 88. 1943.

49. SELYE, H. and STONE, H. Proc. Soc. Exptl. Biol. Med. 52 : 190. 1943.
50. SELYE, H. and STONE, H. Federation Proc. 5 : 93. 1946.
51. SMITH, A. H., MOISE, T. S., and JONES, M. H. Proc. Soc. Exptl. Biol. Med. 24 : 746. 1927.
52. STEENBOCK, H. and NELSON, E. M. J. Biol. Chem. 56 : 355. 1923.
53. SULLIVAN, M. X., HESS, W. C., and SEBRELL, W. H. U.S. Pub. Health Repts. 47 : 75. 1932.
54. SUPPLEE, G. C., BENDER, R. C., and KAHLENBERG, O. J. Endocrinology, 30 : 355. 1942.
55. TEPPERMAN, J., ENGEL, F. L., and LONG, C. N. H. Endocrinology, 32 : 403. 1943.
56. WAINWRIGHT, W. W. and NELSON, M. M. Am. J. Orthodontics, 31 : 406. 1945.
57. WATSON, C. and LYON, G. J. Physiol. 34 : 19. 1906.
58. WILENKO, G. G. Deut. med. Wochschr. 34 : 1897. 1908.

CANADIAN JOURNAL OF RESEARCH

Notes on the Preparation of Copy

GENERAL:—Manuscripts should be typewritten, double spaced, and the **original and one extra copy** submitted. Style, arrangement, spelling, and abbreviations should conform to the usage of this Journal. Names of all simple compounds, rather than their formulae, should be used in the text. Greek letters or unusual signs should be written plainly or explained by marginal notes. Superscripts and subscripts must be legible and carefully placed. Manuscripts should be carefully checked before being submitted, to reduce the need for changes after the type has been set. If authors require changes to be made after the type is set, they will be charged for changes that are considered to be excessive. **All pages, whether text, figures, or tables, should be numbered.**

ABSTRACT:—**An abstract of not more than about 200 words, indicating the scope of the work and the principal findings, is required.**

ILLUSTRATIONS:

(i) *Line Drawings:*—All lines should be of sufficient thickness to reproduce well. Drawings should be carefully made with India ink on white drawing paper, blue tracing linen, or co-ordinate paper **ruled in blue only**; any co-ordinate lines that are to appear in the reproduction should be ruled in black ink. Paper ruled in **green, yellow, or red should not be used** unless it is desired to have all the co-ordinate lines show. Lettering and numerals should be neatly done in India ink preferably with a stencil (**do not use typewriting**) and be of such size that they will be legible and not less than one millimetre in height when reproduced in a cut three inches wide. All experimental points should be carefully drawn with instruments. Illustrations need not be more than two or three times the size of the desired reproduction, but the ratio of height to width should conform with that of the type page. **The original drawings and one set of small but clear photographic copies are to be submitted.**

(ii) *Photographs:*—Prints should be made on glossy paper, with strong contrasts; they should be trimmed to remove all extraneous material so that essential features only are shown. Photographs should be submitted **in duplicate**; if they are to be reproduced in groups, one set should be so arranged and mounted on cardboard with rubber cement; the duplicate set should be unmounted.

(iii) *General:*—**The author's name, title of paper, and figure number should be written in the lower left hand corner (outside the illustration proper) of the sheets on which the illustrations appear.** Captions should not be written on the illustrations, but typed on a separate page of the manuscript. All figures (including each figure of the plates) should be numbered consecutively from 1 up (arabic numerals). **Each figure should be referred to in the text.** If authors desire to alter a cut, they will be charged for the new cut.

TABLES:—Titles should be given for all tables, which should be numbered in Roman numerals. Column heads should be brief and textual matter in tables confined to a minimum. **Each table should be referred to in the text.**

REFERENCES:—**These should be listed alphabetically by authors' names, numbered in that order, and placed at the end of the paper.** The form of literature citation should be that used in the respective sections of this Journal. **Titles of papers should not be given in references listed in Sections A, B, E, and F, but must be given in references listed in Sections C and D.** The first page only of the references cited in papers appearing in Sections A, B, and E should be given. **All citations should be checked with the original articles.** Each citation should be referred to in the text by means of the key number; in Sections C and D the author's name and the date of publication may be included with the key number if desired.

Reprints

The *Canadian Journal of Research* conforms in general with the practice outlined in the *Canadian Government Editorial Style Manual*, published by the Department of Public Printing and Stationery, Ottawa.

Fifty reprints of each paper without covers are supplied free. Additional reprints, if required, will be supplied according to a prescribed schedule of charges. On request, covers can be furnished at cost.